

IN THE SPECIFICATION:Clean Copy of Page 1, Paragraph on Lines 2-3**METHODS FOR DIAGNOSIS AND/OR TREATMENT OF ANTIPHOSPHOLIPID  
ANTIBODY-RELATED DISEASES AND DEVICES THEREFOR**Clean Copy of Page 1, Paragraph on Lines 6-10

The present invention relates to obtaining antibodies recognizing lipids and more particularly, is related to methods for obtaining antibodies against lipidic structures different from the lipidic bilayer, and to the use of these antibodies in diagnostic and/or treatment of diseases associated with the antiphospholipid syndrome; as well as for the determination of physiological states of the cell.

Clean Copy of Page 2, Paragraph on Lines 13-23

On the other hand, some studies have demonstrated that the binding of antiphospholipid antibodies to the lipidic antigen increases in the presence of a plasmatic protein. For example, in 1990, McNeil et al., determined that the binding of antibodies to cardiolipin was markedly enhanced by the plasma protein  $\beta_2$ -glycoprotein I or apoprotein H (*Proc. Nat. Acad. Sci. USA* 87:4120-4124). Additionally, some anti-cardiolipin antibodies are bound directly to  $\beta_2$ -glycoprotein I, as was described by Roubey et al. in 1995 (*J. Immunol.* 154:954-960). These findings suggest that the anti-cardiolipin antibodies may recognize either a cryptic epitope on  $\beta_2$ -glycoprotein I exposed on the complex of  $\beta_2$ -glycoprotein I-cardiolipin, or  $\beta_2$ -glycoprotein I alone but with a very low affinity towards the glycoprotein, as was described by Pengo et al. (1995, *Thromb. Haemost.* 73:29-34).

Clean Copy of Page 6, Paragraph on Lines 15-26

To this respect, the presence in sera from patients with the antiphospholipids syndrome of anti-cardiolipin antibodies, a mitochondrial lipid, of anti-nuclear antibodies and of anti-DNA antibodies, it is indicative of the existence of previous events that cause immunologic damage to cellular membranes, with the disruption of the cells and the exhibition of the intracellular components to the immunologic system, causing the corresponding immunologic reaction that contributes to the development of the syndrome. However, up to now there have not been found

studies which allow to determine the events that cause the disruption of the cellular membrane. In other words, with the existent knowledge so far it is impossible to detect the anti-cardiolipin antibodies, the anti-nuclear or even the anti-DNA antibodies before the damage that has been caused to the cell, impeding an early diagnosis and treatment of the illnesses associated with the syndrome.

**Clean Copy of Page 7, Paragraph on Lines 17-21**

This second hypothesis was proposed in the Master Thesis presented by Monica Lara on August 20, 1999 ("Detection of anti-lipidic particles antibodies in patients with the antiphospholipid syndrome," Master Thesis, Escuela Nacional de Ciencias Biológicas [National School of Biological Sciences], Instituto Politécnico Nacional [National Polytechnic Institute], Mexico.

**Clean Copy of Page 8, Paragraph on Lines 6-12**

Keeping in mind the deficiencies in the structure and in the molecular association of the antigens that are used in the techniques of induction and detection of antiphospholipid antibodies from the methods of the previous techniques, one of the objectives of the present invention consists on using lipidic antigens with a structure and molecular association similar to the one that is present in patients with illnesses associated with antiphospholipid antibodies, with the purpose of providing a method for the detection of anti-lipidic particles antibodies.

**Clean Copy of Page 9, Paragraph on Lines 23-26**

Figures 5A-5C show the cytofluorometric analysis which indicate that the AC15 serum from a patient with primary antiphospholipid syndrome does not show any immunoreaction with liposomal antigens made from DPPC:PC:DPPA (1.2:0.8:1 mole ratio) that lack lipidic particles.

**Clean Copy of Page 9, Paragraph on Lines 30-34**

Figure 7 illustrates the analysis by the cellular-ELISA method of the reaction between sera from patients with the antiphospholipid syndrome and C5337 human pancreas cancer cells; patients sera were used adsorbed and without any adsorption with liposomal antigens made from PC:PA (2:1 mole ratio) bearing lipidic particles induced by calcium.

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**/Clean Copy of Page 11, Paragraph on Lines 17-25**

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Equally, when an antigen that contains lipidic particles is administered in BALB/c mice it has been found surprisingly that these mice developed alopecia and lesions on the face in the form of butterfly wings, similar to those described in some human autoimmune illnesses, as well as deposits of immune complexes and pathological alterations in their different organs. Additionally, it has been also found that these mice firstly developed anti-lipidic particles antibodies and subsequently anti-cardiolipin antibodies, lupus anticoagulant and anti-nuclear antibodies, which confirms that anti-lipidic particles antibodies constitute the first stage in the development of illnesses associated with antiphospholipid antibodies.

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**/Clean Copy of Page 11, Paragraph on Lines 26-34**

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According to the above-mentioned experiments, the presence of anti-lipidic particles antibodies in one of the first stages of the illness, indicates that one of the first events that occurs in the antiphospholipid syndrome is the formation of anti-lipidic particles antibodies. These antibodies when reacting with lipidic particles in cellular membranes, cause damage in these membranes, and finally cells disruption and the exhibition of the intracellular components to the immunitary system; which explains the subsequent presence of anti-cardiolipin antibodies, where cardiolipin is a mitochondrial lipid, of anti-nuclear and anti-DNA antibodies, which have been reported as present in these illnesses in humans.

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Therefore, an aspect of the present invention is to develop a diagnosis method for determining if an individual having clinical characteristics of the primary antiphospholipid syndrome (Table 1), or one of the illnesses associated to the secondary antiphospholipid syndrome (Table 1) and who does not present yet anti-cardiolipin antibodies, lupus anticoagulant, anti-DNA or anti-nuclear antibodies, does have an illness associated to the presence of antiphospholipid antibodies; where such method comprises the steps of detecting in a direct or indirect fashion the presence or absence of lipidic particles in a sample from said individual, and to observe whether lipidic particles are detected or not, where the presence of said lipidic particles indicates the development of an illness associated to the presence of antiphospholipid antibodies in said individual.

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✓ **Clean Copy of Page 17, Paragraph on Lines 29-33**

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- B12
- H) A fifth step of addition and incubation, in which an effective quantity of the peroxidase substrates is added to each one of the wells and said microtiter plate is incubated for 0.1 to 0.5 h at a temperature between 35 and 40°C, stopping the peroxidase reaction by adding an effective quantity of sulfuric acid.
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✓ **Clean Copy of Page 18, Paragraph on Lines 15-18**

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Liposomal-ELISA method allows the simultaneous determination of anti-lipidic particles antibodies in at least 40 sera samples, each one by duplicate, in a single microtiter plate, therefore, this method can be easily applied to the diagnosis of illnesses where this type of antibodies are present.

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- B14
- D) A second step of washing, in which the antigen bound to the antibody porter is washed with a phosphates buffer solution at pH between 7.0 to 7.4. Preferably repeating 3-times and avoiding that the surface of the cellular culture becomes dry when eliminating the phosphates buffer solution.
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In a specific embodiment, the effective quantity of CO<sub>2</sub> is attained with 1 to 10% in volume with regard to air, while the effective quantity of phosphates buffer solution is attained with 1 to 10 ml. The fluorescent substrate can also be selected from the group consisting of phycoerythrin, Cy3 and Percp.

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✓ **Clean Copy of Page 22, Paragraph on Lines 22-26**

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- B16
- H) A fourth step of addition and incubation, in which an effective quantity of peroxidase substrates is added to each one of the wells of microtiter plate, being incubated said plate for 0.1 to 0.5 h at a temperature between 35 to 40°C, stopping the peroxidase reaction by means of an effective quantity of sulfuric acid.
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✓ **Clean Copy of Page 23, Paragraph on Lines 3-5**

B17  
On the other hand in another specific modality of this method, the second antibody can be conjugated to the enzyme alkaline phosphatase, instead of peroxidase, in this case the corresponding alkaline phosphatase substrates are used.

✓ **Clean Copy of Page 23, Paragraph on Lines 6-15**

B18  
Another aspect of the present invention, is to develop an *in vitro* diagnosis instrument for illnesses associated with antiphospholipid antibodies, useful to carry out the method of the present invention. This diagnosis instrument includes at least an indicator reagent to detect the presence of lipidic particles or anti-lipidic particles antibodies in a sample of an individual having clinical characteristics of primary antiphospholipid syndrome (Table 1), or of the illnesses associated to secondary antiphospholipid syndrome (Table 1) and who does not present yet anti-cardiolipin antibodies, lupus anticoagulant, anti-DNA or anti-nuclear antibodies; media to allow the reaction of the sample with the indicator reagent; and, procedures to make evident this reaction.

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In a preferred embodiment, the indicative reagent is selected among liposomes with lipidic particles in their surface, neoplastic cells, anti-lipidic particles polyclonal antibodies, and/or anti-lipidic particles monoclonal antibodies.

✓ **Clean Copy of Page 26, Paragraph on Lines 26-29**

B20  
In a preferred embodiment, the effective quantity of the antigen suspension in the stage A it is of 50 to 100  $\mu$ l. The second antibody can be also conjugated to the enzyme alkaline phosphatase, instead of peroxidase, in this case the corresponding alkaline phosphatase substrates are used.

✓ **Clean Copy of Page 28, Paragraph on Lines 11-14**

B21  
In a preferred embodiment, the antigen suspension is obtained suspending the antigen in a buffer solution at pH between 7.0 to 7.4, in a relationship of 1 to 5 mmole per liter of buffer

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solution for liposomal antigen. Furthermore, fluorescent substrate can also be selected from the group consisting of phycoerythrin, Cy3 and Percp.

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B22  
Costar microtiter plates, with 96 flat-bottom wells with a high lipidic antigens binding property (Costar Co. Cambridge, USA), were coated by the addition of 100  $\mu$ l per well of liposomes made from egg-yolk phosphatidylcholine:phosphatidate (2:1 mole ratio) in Tris-NaCl buffer (10 mM, 1 mM) pH 7, containing 0.1  $\mu$ mol of phosphatidate, and treated with 5 mM  $\text{CaCl}_2$  to induce lipidic particle formation. Microtiter plates were incubated 1 h at room temperature. After microtiter plates were incubated they were blocked for 1 h at room temperature by addition of 200  $\mu$ l per well of 0.4% (w/v) gelatin in Tris-NaCl buffer (10 mM, 1 mM) pH 7, containing  $\text{CaCl}_2$  5 mM. Then the blocking solution was discarded by suction and 100  $\mu$ l of human sera, from patients with the antiphospholipid syndrome, at 1:50 dilution using blocking solution were quickly added to each well in duplicate, to avoid that these wells becomes dry; all solutions were added subsequently in the same way. As a positive control, the supernatant of a hybridoma containing a monoclonal antibody against lipidic particles, from IgM isotype, at 1:100 dilution using blocking solution were added to four wells. Human sera were heated previously at 56°C for 30 min for the inactivation of the complement. After microtiter plates were incubated 1h at room temperature they were washed 4-times with 500  $\mu$ l of blocking solution. Then 100  $\mu$ l of peroxidase-conjugated goat anti-Fc of human IgG, IgA and IgM antibodies or anti-Fc of mouse IgM antibodies at 1:2000 dilution into blocking solution were added to each well, respectively, as second antibody. After 1 h of incubation at room temperature microtiter plates were washed 4-times again with the blocking solution and 100  $\mu$ l of freshly prepared peroxidase substrates were added to each well (10 mg o-phenylendiamine, 25 ml Tris-NaCl buffer (10 mM, 1 mM) pH 7, and 20  $\mu$ l of 30%  $\text{H}_2\text{O}_2$ ) and allowed to incubate in an oven at 37°C for 20 min. Enzyme reaction was stopped by addition of 50  $\mu$ l per well of 2.5 M sulfuric acid. Absorbances were read at 492 nm in an ELISA Labsystems reader Multiskan MS model; duplicate values were averaged for each serum sample tested.

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**Clean Copy of Page 30, Paragraph on Lines 22-27**

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B23 Anti-cardiolipin antibodies were detected using cardiolipin coated to ELISA microtiter plates as antigen (Loizou et al., 1985, *Clin. Exp. Immunol.* 62:738-745). Results are also expressed in Arbitrary Units (AU) and they are considered positive when they have values  $\geq 1.9$  AU for IgG isotype, and  $\geq 2.4$  AU for IgM isotype (Loizou et al., *op. cit.*, 1985). All patients' sera were positive for IgG isotype and some of them were positive for IgM isotype (Table 2).

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**Table 2.** Detection of anti-cardiolipin and anti-lipidic particles antibodies in human sera

Healthy blood donors	Anti-cardiolipin Antibodies (ELISA)	Anti-lipidic particles Antibodies (liposomal antigen made from phosphatidylcholine: phosphatidate (2:1) + CaCl <sub>2</sub> ) (Cytofluorometry)	Patients' sera and diagnostic	Anti-cardiolipin antibodies (ELISA)		Anti-lipidic particles antibodies (liposomal antigen made from phosphatidylcholine: phosphatidate (2:1) + CaCl <sub>2</sub> ) (Cytofluorometry)	
	IgM (+) ≥ 2.4 AU	IgG (+) ≥ 1.9 AU		IgM (+) ≥ 2.4 AU	IgG (+) ≥ 1.9 AU	Positive results at: (+) D≥0.5, p<0.001	
1H	-	-	AC11 PAPS	-	7.5	Polyvalent	IgM
2H	-	-	AC12 PAPS	-	56.3	D=0.77	IgG
3H	-	-	AC13 SLE	5.24	17.2	D=.77	D=0.65
4H	-	-	AC14 SLE	-	10.6	D=0.74	D=0.62
5H	-	-	AC15 PAPS	-	6.7	D=0.74	D=0.84
6H	-	-	AC16 SLE	-	2.52	D=0.75	D=0.59
7H	-	-	AC17 SLE	-	4.3	D=0.75	D=0.59
8H	-	-	AC18 SLE + APS	-	67.4	D=0.73	D=0.63
9H	-	-	AC19 SLE	-	13.6	D=0.73	D=0.73
10H	-	-	AC20 SLE	-	9.3	D=0.75	D=0.73
11H	-	-	AC21 PAPS	-	3.36	D=0.75	D=0.62
12H	-	-	AC22 SLE + APS	2.8	15.4	D=0.56	D=0.52
13H	-	-	AC23 SLE + APS	-	19.2	D=0.59	D=0.61
14H	-	-	AC24 PAPS	-	18.0	D=0.61	D=0.61
15H	-	-	AC25 PAPS	3.95	16.3	D=0.53	D=0.62
16H	-	-	AC26 SLE	3.06	9.2	D=0.53	D=0.62
17H	-	-	AC27 PAPS	-	8.6	D=0.51	D=0.54
18H	-	-	AC28 PAPS	-	11.5	D=0.51	D=0.54
19H	-	-	AC29 SLE	-	11.08	D=0.43	D=0.57
20H	-	-	AC30 PAPS	-	14.7	N/D	D=0.57
21H	-	-	AC31 SLE + APS	-	19.4	D=0.66	D=0.50
22H	-	-	AC32 SLE + APS	3.0	39.6	D=0.56	D=0.57
23H	-	-	AC33 PAPS	-	23.7	D=0.56	D=0.54
24H	-	-	AC34 SLE + APS	-	34.4	D=0.56	D=0.74
25H	-	-	AC35 PAPS	4.0	18.0	D=0.66	D=0.75
26H	-	-	AC36 SLE	44.0	158.0	D=0.64	D=0.64
27H	-	-	AC37 SLE	-	11.0	D=0.64	D=0.75
28H	-	-	AC38 PAPS	3.0	2.0	D=0.64	D=0.75
29H	-	-	AC39 SLE + APS	-	52.0	D=0.64	D=0.75
30H	-	-	AC40 SLE	4.0	18.0	D=0.66	D=0.75

PAPS - Primary antiphospholipid syndrome. SLE - Systemic lupus erythematosus. APS + SLE - Antiphospholipid syndrome secondary to systemic lupus erythematosus

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✓ **Clean Copy of Page 33, Paragraph on Lines 5-8**

B25 Patients' sera and sera from healthy blood donators were supplied us by Dr. Carlos Lavalle Montalvo, Manager of the Infectology Hospital of the Medical Center "La Raza", from México, D. F., México.

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✓ **Clean Copy of Page 33, Paragraph on Lines 11-25**

B26 Reaction of human sera, from healthy blood donators or from patients with the antiphospholipid syndrome, with liposomal antigens made from egg-yolk phosphatidylcholine:phosphatidate (2:1 mole ratio) in Tris-NaCl buffer (10 mM, 1 mM) pH 7, containing 0.1  $\mu$ mol of phosphatidate, and treated with 5 mM  $\text{CaCl}_2$  to induce lipidic particles formation, is showed in Figure 1. Immunoreaction of patients sera with the lipidic particles was clearly different from that of healthy blood donators sera, or control sera, since the reaction with the peroxidase substrates was negative when control sera were used. In general, control serum gave values smaller than one AU. All the values from control sera were combined to obtain the arithmetic mean and the standard deviation. We then consider as positive all results greater than 3 standard deviations from the mean. After this analysis, sera from the 30 healthy blood donors were mixed and the mixture was used as a control sera for subsequent analysis. In Figure 1 the dark line indicates the upper limit above which the reactions of sera with lipidic antigens are positive. The reaction of most patients sera was clearly positive, with values of AU higher than 6.

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✓ **Clean Copy of Page 34, Paragraph on Lines 12-23**

B27 Example 1 was repeated but using as antigens "rigid" liposomes made from dipalmitoylphosphatidylcholine:egg-yolk phosphatidylcholine:dipalmitoylphosphatidate (1.2:0.8:1.0 mole ratio) in Tris-NaCl buffer (10 mM, 1 mM) pH 7, which when incubated with 5 mM  $\text{BaCl}_2$  they conserve the smooth surface of bilayer. In this case, the reaction of the substrates of peroxidase-conjugated to the second antibody was negative. Because liposomes did not have any lipidic particles, therefore the anti-lipidic particles antibodies did not react with them and consequently the second antibody did not bind these liposomes, which explains the negative reaction of peroxidase substrates. These results discard the possibility that anti-lipidic particles antibodies recognize a lipid-divalent cation complex and/or the reduction in the

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liposomal surface charge due to the binding of divalent cations without affecting the bilayer lipid arrangements and their change to lipidic particles arrangements (Aguilar et al., *op. cit.*, 1999).

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**Clean Copy of Page 34, Paragraph on Line 28 through Page 35 Line 6**

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Example 1 was repeated with some modifications. In this experiment liposomes made from egg yolk phosphatidylcholine:phosphatidate (2:1 mole ratio) in Tris-NaCl buffer (10 mM, 1 mM) pH 7, containing 0.1  $\mu$ mol of phosphatidate, and treated with 5 mM  $\text{CaCl}_2$  to induce lipidic particles formation, were incubated directly with peroxidase-conjugated goat anti-Fc of human IgG, IgA and IgM antibodies, or with the supernatant of a hybridoma producing unrelated monoclonal antibodies, as those against a membranal protein of *Trichinella spiralis*, from IgM isotype, and peroxidase-conjugated goat anti-Fc of mouse IgM antibodies. In both cases, the reaction with peroxidase substrates was negative, because in absence of human anti-lipidic particles antibodies or mouse anti-lipidic particles monoclonal antibody the second antibody, peroxidase-conjugated goat anti-Fc of human IgG, IgA and IgM or anti-Fc of mouse IgM antibodies, do not bind directly to lipidic particles induced by calcium in liposomal antigens.

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**Clean Copy of Page 35, Paragraph on Lines 11-17**

B29  
Example 1 was repeated but in the absence of liposomal antigens, in consequence the reaction of substrates of the peroxidase-conjugated to second antibody was negative. Due to anti-lipidic particles antibodies do not bind directly to microtiter plate which could give a false positive result, because microtiter plate was blocked with the gelatin that is used in this methodology, consequently the second antibody do not bind to microtiter plate which explains the negative reaction of the substrates of peroxidase-conjugated to the second antibody.

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**Clean Copy of Page 36, Paragraph on Lines 18-31**

B30  
Autofluorescence histograms obtained from egg yolk phosphatidylcholine: phosphatidate (2:1 mole ratio) liposomes showed values between 1 to 10 fluorescence units (a, Fig. 2A). The detection of liposomal autofluorescence allowed the application of cytofluorometry to the analysis of immunologic reactions where liposomal antigens are used. Liposomal autofluorescence (a, Fig. 2A) was not modified when liposomes were incubated with 5 mM  $\text{CaCl}_2$  (c, Fig. 2B), which indicates that the presence of lipidic particles in liposomes did not

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modify the liposomal autofluorescence. Furthermore, this fluorescence was not modified by the addition of FITC-conjugated goat anti-Fc of human IgG, IgA and IgM or anti-Fc of mouse IgM antibodies as second antibodies, which indicates that these antibodies do not bind directly liposomal antigens, and therefore they can not produce a false positive reaction. Results with the FITC-conjugated goat anti-Fc of human IgG, IgA and IgM antibodies as second antibody at 1:200 final dilution are shown in: b, Fig. 2A and d, Fig. 2B, with liposomal antigens in absence of calcium (b, Fig. 2A) as in presence of this divalent cation (d, Fig. 2B).

✓ **Clean Copy of Page 36, Paragraph on Line 32 through Page 37 Line 4**

B30

Similar results were obtained with liposomes made from phosphatidylcholine; phosphatidylcholine:cardiolipin (2:1 mole ratio); phosphatidylcholine:phosphatidylserine (4:1 mole ratio) or from dipalmitoylphosphatidylcholine:egg-yolk phosphatidylcholine:dipalmitoylphosphatidate (1.2:0.8:1 mole ratio), respectively. Therefore the cytofluorometry can be applied in general to the analysis of immunologic reactions where liposomal antigens with different lipidic formulations are used.

✓ **Clean Copy of Page 37, Paragraph on Lines 26-33**

B32

Fluorescence readings were made in the FL-1 channel. The relative size and/or liposomal aggregation were analyzed by diffraction of the laser beam in the FSC (forward scatter light) channel and the granularity or liposomal bilayers complexity was analyzed by refraction and reflection of the laser in the SSC (side scatter light) channel. Analysis of 10,000 liposomes was made in a logarithmic scale with the following detectors: FSC in E00, with a detector compensation threshold of 52 V; SSC of 401 V and FL-1 of 748 V (Baeza et al., *op. cit.*, 1995). The obtained data were analyzed with the Cellquest program (Beckton Dickinson).

✓ **Clean Copy of Page 38, Paragraph on Lines 5-21**

B33

H308 monoclonal antibody do not show any reactivity with bilayer lipid arrangements, since the fluorescence detected from smooth liposomes incubated with this monoclonal antibody (a, Fig. 3A) was similar to the autofluorescence of control liposomes, in Tris-NaCl or treated with calcium, that were not incubated with monoclonal antibody (g, h, Fig. 3D). Furthermore, SSC and FSC values indicated the absence of lipidic particles and of liposomal aggregation in

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smooth liposomes that were not treated with calcium (i, Fig. 3E; k, Fig. 3F) no matter if they were incubated with H308 monoclonal antibody (c, Fig. 3B; e, Fig. 3C). On the contrary, the 60-fold increase in the fluorescence of liposomes treated with calcium (b, Fig. 3A) with regard to the fluorescence of liposomes with lipids in bilayers (a, Fig. 3A), with a value in the fluorescence difference among these liposomal populations in a logarithmic scale ( $D$ )=0.9 at  $p < 0.001$ , showed the reaction of H308 monoclonal antibody with the lipidic particles induced by calcium. Values of  $D \geq 0.5$  at  $p < 0.001$  indicate a difference among the studied populations that is highly significant from the statistical point of view (Lampariello, 2000, *Cytometry* 39:179-188). Therefore, values of  $D \geq 0.5$  at  $p < 0.001$  were considered as positive results and indicative of the presence of anti-lipidic particles antibodies in the analyzed samples.

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✓ **Clean Copy of Page 38, Paragraph on Lines 30-34**

B34

Monoclonal antibody reaction with lipidic particles of liposomal antigens is considered as a positive reference of the reaction of patients' antibodies with this type of lipidic structures. In consequence it is necessary to include this determination as a positive control in the analysis of the detection of anti-lipidic particles antibodies in sera from human individuals or animals by liposomal cytofluorometry.

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✓ **Clean Copy of Page 40, Paragraph on Lines 5-11**

B35

Fluorescence readings were made in the FL-1 channel. The relative size and/or liposomal aggregation were analyzed in the FSC channel and the granularity or liposomal bilayers complexity was analyzed in the SSC channel. Analysis of 10,000 liposomes was made in a logarithmic mode with the following detectors: FSC in E00, with a detector compensation threshold of 52 V; SSC of 401 V and FL-1 of 748 V (Baeza et al., *op. cit.*, 1995). The obtained data were analyzed with the Cellquest program (Beckton Dickinson).

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✓ **Clean Copy of Page 40, Paragraph on Lines 12-16**

B36

As a negative control, the reaction of healthy blood donators sera with liposomes made from egg yolk phosphatidylcholine:phosphatidate (2:1 mole ratio) in Tris-NaCl buffer (10 mM, 1 mM) pH 7, containing 0.1  $\mu$ mol of phosphatidate, and treated with 5 mM  $\text{CaCl}_2$  to induce

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*cont*  
lipidic particles formation was analyzed. FITC-conjugated goat anti-Fc of human IgG, IgA and IgM antibodies were used as second antibody.

*B37*  
Clean Copy of Page 40, Paragraph on Lines 17-27

Thirty healthy blood donors' sera were studied. These sera did not present any immunoreaction with lipidic particles, since fluorescence graphs obtained with liposomes incubated with them were similar to those of control liposomes that were exclusively incubated with 5 mM  $\text{CaCl}_2$  (h, Fig. 3D; and j, Fig. 3E from Example 2A). After this analysis, sera from the 30 healthy blood donors were mixed and the mixture was used as a control sera for subsequent analysis. Cytofluorometry graphs of mixed sera are presented in: a, Figs. 4A, D, G, J, M, P, S and V; in c, Figs. 4B, E, H, K, N, Q, T and W, and in e, Figs. 4C, F, I, L, O, R, U and X. FSC values (e, Figs. 4C, F, I, L, O, R, U and X) showed the absence of liposomal aggregation by the treatment with healthy blood donors sera, because they were very similar to those of liposomes control in absence of human sera indicated in: l, Fig. 3F, from Example 2A.

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Clean Copy of Page 40, Paragraph on Line 28 through Page 41 Line 11

Immunoreaction of all patients' sera with liposomal antigens treated with calcium showed a fluorescence 20 to 40-fold higher than that of control sera reaction, with a difference between liposomal fluorescence in a logarithmic scale ( $D \geq 0.5$  at  $p < 0.001$  (Table 2). Values of  $D \geq 0.5$  at  $p < 0.001$  were considered as positive results and indicative of the presence of anti-lipidic particle antibodies in sera analyzed, in a similar way as it was described for H308 monoclonal antibodies. As example, fluorescence histograms of eight sera from patients with systemic lupus erythematosus (SLE) (AC19 and AC20), with primary antiphospholipid syndrome (PAPS) (AC15, AC21 and AC30) or with antiphospholipid syndrome secondary to systemic lupus erythematosus (SLE+APS) (AC18, AC22 and AC31) are shown in: b, Fig. 4A; g, Fig. 4D; j, Fig. 4G; m, Fig. 4J; o, Fig. 4M; r, Fig. 4P; u, Fig. 4S; and x, Fig. 4V. In the eight sera the reaction between anti-lipidic particle antibodies contained in patients' sera and lipidic particles of liposomal antigens although positive, were clearly different from each other and with regard to the reaction of H308 monoclonal antibody (compare d, Fig. 4B; h, Fig. 4E; k, Fig. 4H; n, Fig. 4K; p, Fig. 4N; s, Fig. 4Q; v, Fig. 4T; and y, Fig. 4W with d, Fig. 3B, from Example 2A), which can be attributed to the polyclonal origin of human antibodies.

**Clean Copy of Page 42, Paragraph on Lines 4-12**

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"Rigid" liposomes made from dipalmitoylphosphatidylcholine:egg-yolk phosphatidylcholine:dipalmitoylphosphatidate (1.2:0.8:1.0 mole ratio) in Tris-NaCl buffer (10 mM, 1 mM) pH 7, that incubated with BaCl<sub>2</sub> 5 mM conserved the smooth surface, were used as antigens. In "rigid" liposomes there were not the formation of lipidic particles because their rigid bilayers do not allow the lipidic movement that is required to form lipidic particles. These liposomes were incubated with sera from patients with the antiphospholipid syndrome and FITC-conjugated goat anti-Fc of human IgG, IgA and IgM antibodies were used at a final dilution of 1:200 as second antibody.

**Clean Copy of Page 45, Paragraph on Lines 11-18**

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Fluorescence readings were made in the FL-1 channel. Plaquettes relative size and/or plaquettes aggregation were analyzed by diffraction of the laser beam in the FSC channel. Granularity or membranal plaquette complexity was analyzed by refraction and reflection of the laser in the SSC channel. Analysis of 10,000 plaquettes was made with the following detectors: FSC in E00, in a lineal mode with an amplifier gain of 5 V and with a detector compensation threshold of 52 V; SSC of 450 V and FL-1 of 700 V, both in logarithmic mode (Baeza et al., *op. cit.*, 1995). The obtained data were analyzed with the Cellquest program (Beckton Dickinson).

**Clean Copy of Page 46, Paragraph on Lines 11-29**

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C5337 pancreas cancer cells were used as antigens, then  $1 \times 10^5$  cells was seeded in each well of a flat-bottom 96-wells microtiter plates, and they were incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub> until cell confluence in the wells reached 100%. After incubation, 200 µl of a blocking solution containing Tris-NaCl buffer (10 mM, 135 mM) pH 7, and 5% fetal calf serum, were added to each one of the wells and microtiter plates were incubated for 30 min at 37°C. Additionally, the blocking solution was eliminated and 100 µl of sera from patients with the antiphospholipids syndrome, or from healthy blood donators at 1:50 final dilution, using blocking solution, were quickly added to avoid that cells surface becomes dry. All solutions were added subsequently in the same way. After cell cultures were incubated for 30 min at 37°C in an atmosphere containing 5% CO<sub>2</sub>, they were washed 3-times with 200 µl of blocking solution for 5 min in each washing. Next, 100 µl of peroxidase-conjugated goat anti-Fc of human

B41  
cont

IgG, IgA and IgM antibodies at 1:2000 dilution, into blocking solution, were added as second antibody. Microtiter plates were incubated for 30 min at 37°C in an atmosphere containing 5% CO<sub>2</sub>. After incubation, microtiter plates were washed as it was indicated and 100 µl of peroxidase substrates were added to each one of the wells and plates were again incubated for 20 min at 37°C. Finally 50 µl of 2.5 M sulfuric acid were added to stop the peroxidase reaction and the absorbency was read at 492 nm in an ELISA Labsystems reader Multiskan MS model.

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✓ **Clean Copy of Page 47, Paragraph on Lines 27-31**

B42

This detection was carried out as it was indicated in the Example 3, with the difference that C5337 pancreas cancer cells were incubated with sera from patients with the antiphospholipid syndrome bearing anti-lipidic particles antibodies instead of H308 monoclonal antibody. Patients' sera were used at 1:50 dilution and FITC-conjugated goat anti-Fc of human IgG, IgA and IgM antibodies were used as second antibody.

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✓ **Clean Copy of Page 47, Paragraph on Line 33 through Page 48 Line 2**

B43

Neoplastic cell cultures were marked with the anti-lipidic particles antibodies from patients' sera in a similar way as it was described for C5337 pancreas cancer cells in Figure 6, from Example 3, showing the binding of lipidic particles from neoplastic membranes with these anti-lipidic particles antibodies.

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✓ **Clean Copy of Page 48, Paragraph on Lines 3-6**

B44

On the other hand, the methodology described in this Example can be applied in an alternative way to the detection of anti-lipidic particles antibodies in patients' sera when these antibodies have been not yet detected by the procedures indicated in the Examples 1 and 2B.

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✓ **Clean Copy of Page 48, Paragraph on Lines 13-15**

B45

In this preferred embodiment of the diagnosis kit, the cell samples coming from the ill individual are made react with anti-lipidic particles monoclonal antibody, in other words, with the indicator reagent.

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✓ **Clean Copy of Page 48, Paragraph on Lines 23-27**

B46 In another preferred embodiment of the present invention, a kit for the detection of lipidic particles in cells in different physiologic states coming from a human or animal subject, includes: at least an indicator reagent including at least an anti-lipidic particles monoclonal antibody; at least a buffer solution as a medium to allow the reaction; and fluorescent or enzymatic procedures to make evident this reaction.

✓ **Clean Copy of Page 49, Paragraph on Lines 16-23**

B47 Immunoreaction analysis of mice sera was made by the liposomal cytofluorometry method. Egg-yolk phosphatidylcholine:phosphatidate liposomes (2:1 molar ratio) in Tris-NaCl buffer (10 mM, 1 mM) pH 7, containing 0.1  $\mu$ mol of phosphatidate, and treated with 5 mM  $MnCl_2$  to induce lipidic particles formation were used as antigens. Analysis of 10,000 liposomas was made in logarithmic mode with the following detectors: FSC in E00, with a detector compensation threshold of 52V; SSC of 401 V and FL-1 of 748 V (Baeza et al., *op. cit.*, 1995). The obtained data were analyzed with the Cellquest program (Beckton Dickinson).

✓ **Clean Copy of Page 50, Paragraph on Lines 6-16**

B48 Sera from mice after they were immunized with liposomal antigens treated with manganese showed an immunoreaction that produce a liposomal fluorescence 10 to 100-fold higher than the reaction of mice control sera (Fig. 8C), with values of  $D \geq 0.5$  at  $p < 0.001$ . As example, cytofluorometry graphs of the reaction of serum from RB11, RB14 and RB17 mice are showed in Figs. 8 D, 8E and 8F. Reaction between the antibodies of sera from these mice and lipidic particles although positive, was different for each serum, with values of  $D=0.9$ ,  $D=0.91$  and  $D=0.79$ , respectively, which can be attributed to the polyclonal origin of these antibodies. SSC values from immunoreaction (Figs. 8 D, 8E and 8F) were similar to those of liposomes control incubated with manganese (Fig. 8 A), and they showed the presence of lipidic particles which give the reaction with the anti-lipidic particles antibodies from mice sera.

✓ **Clean Copy of Page 51, Paragraph on Lines 13-18**

B49 Immunoreaction analysis of mice sera was made by the liposomal cytofluorometry method as it was indicated in Example 4. Egg-yolk phosphatidylcholine:phosphatidate liposomes



B49  
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(2:1 molar ratio) in Tris-NaCl buffer (10 mM, 1 mM) pH 7, containing 0.1  $\mu$ mol of phosphatidate, and treated with 8 mM procainamide to induce lipidic particles formation were used as antigens. Analysis of 10,000 liposomas was made in logarithmic mode as it was described in Example 4.

✓ **Clean Copy of Page 52, Paragraph on Lines 3-14**

B50

Sera obtained after mice were immunized with liposomal antigens treated with the lipidic particles inducer drug procainamide showed an immunoreaction that produce a liposomal fluorescence 10 to 100-fold higher than the reaction of control mice sera (Fig. 10C), with values of  $D \geq 0.5$  at  $p < 0.001$ . As example, cytofluorometry graphs of the reaction of serum from RF11, RF14 and RF17 mice are showed in Figs. 10D, 10E and 10F, respectively. Reaction between the antibodies of sera from these mice and lipidic particles although positive, was different for each serum, with values of  $D=0.8$ ,  $D=0.72$  and  $D=0.67$ , respectively, which can be attributed to the polyclonal origin of these antibodies. SSC values from immunoreaction (Figs. 10D, 10E and 10F) were similar to those of liposomes control incubated with procainamide (Fig. 10A), and they showed the presence of lipidic particles in liposomes which give the reaction with the anti-lipidic particles antibodies from mice sera.

**Clean Copy of Page 52, Paragraph on Line 24 through Page 53 Line 2**

B51

After the detection of anti-lipidic particles antibodies in immunized mice anti-cardiolipin antibodies, anti-nuclear and anticoagulant antibodies were also detected in them, in a similar fashion as it was described for mice immunized with liposomes treated with manganese, from Example 4. These findings corroborate that anti-lipidic particles antibodies constitute the first stage in the development of illnesses associated with antiphospholipid antibodies. Furthermore, in mice immunized with liposomes incubated with procainamide or chlorpromazine it has been demonstrated the presence of deposits of immune complexes in different organs. In addition, these mice developed alopecia and lesions in the face in the form of butterfly wings similar to those that have been described in human systemic lupus erythematosus. In Figure 11 the picture of a 7-months age BALB/c female mouse that was treated with liposomes bearing lipidic particles induced by chlorpromazine, where alopecia and lesions in the face in the form of butterfly wings can be observed.

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✓ **Clean Copy of Page 53, Paragraph on Lines 24-29**

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B52 Immunoreaction analysis of mice sera was made by the liposomal cytofluorometry method as it was indicated in Example 4. Egg-yolk phosphatidylcholine:phosphatidate liposomes (2:1 molar ratio) in Tris-NaCl buffer (10 mM, 1 mM) pH 7, containing 0.1  $\mu$ mol of phosphatidate, and treated with 8 mM procainamide to induce lipidic particles formation were used as antigens. Analysis of 10,000 liposomas was made in logarithmic mode as it was described in Example 4.

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✓ **Clean Copy of Page 54, Paragraph on Lines 1-12**

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B53 Sera obtained from mice after they were treated with the lipidic particles inducer drug procainamide showed an immunoreaction that produce a liposomal fluorescence 4-fold higher than the reaction of mice sera before treatment (a, Figs. 12 A, D, and G), with values of  $D \geq 0.5$  at  $p < 0.001$ . As example, cytofluorometry graphs of the reaction of serum from RP37, RP38 and RP39 mice are showed in: b, Fig. 12 A; g, Fig. 12 D; and j, Fig. 12 G. Reaction between the antibodies of sera from these mice and lipidic particles although positive, was different to each serum, with values of  $D=0.58$ ,  $D=0.68$  and  $D=0.8$ , respectively, which can be attributed to the polyclonal origin of these antibodies. SSC values from immunoreaction showed in: d, Fig. 12 B; h, Fig. 12E; and k, Fig. 12H were similar to those of liposomes control incubated with procainamide (Fig. 10A), and they showed the presence of lipidic particles which give the reaction with the anti-lipidic particles antibodies from mice sera.

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✓ **Clean Copy of Page 54, Paragraph on Lines 18-22**

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B54 Anti-lipidic particles antibodies were also detected before than anti-cardiolipin antibodies, anti-nuclear and anticoagulant antibodies in these mice, in a similar way as it was described for mice in Examples 4 and 4A. Furthermore, the presence of deposits of immune in different organs and the development of alopecia and lesions in the face in the form of butterfly wings were also showed in these mice.

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**Clean Copy of Page 55, Paragraph on Lines 12-17**

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B55 Immunoreaction analysis of mice sera was made by the liposomal cytofluorometry method as it was indicated in Example 4. Egg-yolk phosphatidylcholine:phosphatidate liposomes

B55  
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(2:1 molar ratio) in Tris-NaCl buffer (10 mM, 1 mM) pH 7, containing 0.1  $\mu$ mol of phosphatidate, and treated with 5 mM  $\text{CaCl}_2$  to induce lipidic particles formation were used as antigens. Analysis of 10,000 liposomas was made in logarithmic mode as it was described in Example 4.

✓ **Clean Copy of Page 55, Paragraph on Lines 29-33**

B56

Anti-lipidic particles antibodies were also detected before than anti-cardiolipin antibodies, anti-nuclear and anticoagulant antibodies in these mice, in a similar way as it was described for mice in Examples 4, 4A, and 4B. Additionally, the presence of deposits of immune complexes in different organs and the development of alopecia and lesions in the face in the form of butterfly wings were also showed in these mice.

**Clean Copy of Page 56, Paragraph on Line 20 through Page 57 Line 2**

B57

The spleen of the RB14 BALB/c female mouse producing the higher titers of anti-lipidic particles antibodies was removed under sterility conditions and it was placed in a petri dish with 6 ml of incomplete DMEM cell culture medium. Spleen mouse was dispersed until a suspension of single cells was obtaining using blunt tips pincers. Cellular suspension was transferred to a 15-ml falcon tube and it was left in repose so that the thick residuals settle down. Next, cellular suspension was transferred to another falcon tube and it was centrifuged at 17 x g for 7 min. Subsequently the supernatant was decanted and cellular pellet was resuspended by gently agitation and cellular suspension was diluted by the addition, drop by drop, of 10 ml of incomplete DMEM cell culture medium. Cellular suspension was centrifuged as it was already indicated, then the supernatant was decanted and 4 ml of 0.16 M  $\text{NH}_4\text{Cl}$  were added for erythrocytes lysis. In this step the tube containing cellular suspension was incubated at 37°C and it was gently rotated during 4 min. Later on 6 ml of incomplete DMEM cell culture medium was added and cellular suspension was centrifuged at 17 x g for 7 min. After centrifugation the supernatant was decanted and cellular pellet was gently resuspended in 10 ml of incomplete DMEM cell culture and was allowed to stand at room temperature until their were used (Köhler and Milstein, 1975. Nature 256:495.497).

**Clean Copy of Page 57, Paragraph on Lines 3-27**

B58

On the other hand, P3x63Ag8U.1 myeloma cells were collected from cell culture plates and transferred to falcon tubes. Aliquots from P3x63Ag8U.1 myeloma cells and mouse spleen cells were treated with trypan blue and they were counted in a Neubauer camera. The viability of both cellular suspension were higher than 95%. P3x63Ag8U.1 myeloma cells and mouse spleen cells were mixed in a 1:1 cellular proportion, using  $36 \times 10^6$  cells of each cellular type, later cellular mixture was washed with 10 ml of incomplete DMEM cell culture medium. After centrifugation at  $17 \times g$  for 5 min the supernatant was decanted and cellular pellet was gently resuspended. Subsequently, 1 ml of 4000 polyethyleneglycol solution was added drop by drop, during 1 min, and the mixture was manually shaken up for 1.5 min, then 1 ml of incomplete DMEM cell culture medium was added for 30 sec with slow tube rotation. Next, 3 ml of incomplete DMEM cell culture medium was added for 30 sec also with slow tube rotation, later 16 ml of the same medium was added for 1.5 min with gently agitation. Finally the volume of the fused cell suspension was completed to 40 ml with incomplete DMEM cell culture medium and fused cell suspension was incubated without agitation for 5 min at room temperature. Later on fused cell suspension was centrifuged at  $17 \times g$  for 5 min, the supernatant was decanted and fused cell pellet was washed again with 40 ml of incomplete DMEM medium. Fused cell pellet was resuspended in 30 ml of selection DMEM-HAT medium and aliquots of 100  $\mu$ l of this fused cell suspension were seeded in each one of the wells of three 96-wells flat-bottom microtiter plates which 24 hs before cell fusion were seeded with macrophages as feeder cells. Microtiter plates were incubated at 37°C, in an atmosphere with 5% of CO<sub>2</sub>. After five or eight days of the cellular fusion hybridomas were fed with 50  $\mu$ l of selection DMEM-HAT medium, finally after 11 days of the cellular fusion hybridoma supernatants were changed by 100  $\mu$ l of DMEM-HAT media.

**Clean Copy of Page 57, Paragraph on Line 28 through Page 58 Line 4**

B59

After hybridomas growing the supernatants were screened by the liposomal-ELISA method in order to detect the production of anti-lipidic particles antibodies by them. Cellular samples from all hybridomas producing anti-lipidic particles monoclonal antibodies were frozen at -70°C in liquid nitrogen. Later, 10 hybridomas with high anti-lipidic particles monoclonal antibodies titers were chosen (Table 3) and they were cloned again by limiting dilution in 96-

B59  
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wells flat-bottom microtiter plates. After hybridomas growing supernatants were screened again by the liposomal-ELISA method and those producing the higher titers of anti-lipidic particles monoclonal antibodies were cultivated in 250 ml bottles for the massive obtention of supernatants containing these antibodies.

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**Clean Copy of Page 58, Paragraph on Lines 12-22**

B60

Costar microtiter plates, with 96 flat-bottom wells with a high lipidic antigens binding property (Costar Co. Cambridge, USA) were coated by the addition to each one of the wells of 100  $\mu$ l of liposomes made from egg-yolk phosphatidylcholine:phosphatidate (2:1 molar ratio) in Tris-NaCl buffer (10 mM, 1 mM) pH 7, containing 0.1  $\mu$ mol of phosphatidate, and treated with 5 mM  $\text{CaCl}_2$  to induce lipidic particles formation. Microtiter plates were incubated at room temperature for 1 h and they were blocked for 1 h at room temperature in a similar way as was described in Example 1. Next, blocking solution was discarded by suction and 100  $\mu$ l of H308 monoclonal antibody that was previously incubated with the phosphorylated haptens were added immediately to each one of the wells, to avoid that they becomes dry off.

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**Clean Copy of Page 59, Paragraph on Lines 3-6**

B61

Aliquots of 100  $\mu$ l of H308 monoclonal antibody were incubated with 100  $\mu$ l of each one of the hapten solutions for 30 min at 30°C. Later on, the liposomal-ELISA method was applied as it was described in Example 1. Peroxidase-conjugated goat anti-Fc of mouse IgM antibodies were used as second antibody.

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**Clean Copy of Page 59, Paragraph on Lines 17-28**

B62

Inhibition of H308 monoclonal antibody reaction with phosphorylcholine and glycerolphosphorylcholine indicate that the antigen recognition domain in H308 monoclonal antibody has subdomains that recognize specifically the choline methyl groups which lacks ethanolamine and serine (Fig. 13). In addition, total immunoreaction inhibition attained by glycerolphosphorylcholine suggests that the antigen domain that recognize H308 monoclonal antibody include chemical groups of glycerol. These findings are in agreement with the structural pattern proposed for the lipidic particle (Cullis et al., *op. cit.*, 1991) (Fig. 15) where monolayer lipids (C, Fig. 15) that recover the molecular arrangement different to bilayer (B, Fig. 15) are

B62  
coma  
more separate than lipids that constitute a normal monolayer (A, Fig. 15). In an open monolayer (C, Fig. 15) glycerolphosphorylcholine is more exposed than in a normal bilayer therefore this is the region in which the H308 monoclonal antibody reacts.

**Clean Copy of Page 59, Paragraph on Line 30 through Page 60 Line 4**

B63  
Possibly the central domain of lipidic particle, the region that is observed as inverted micella in: B, Fig. 15, is formed by conic shaped lipids such as phosphatidate. In contrast, monolayers most open than a normal monolayer would be formed by phosphatidylcholine and they would be the regions that identify the H308 monoclonal antibody. If H308 monoclonal antibody reacts specifically with a phosphatidylcholine open monolayer, is clear that this antibody does not show any immunoreaction with liposomes formed exclusively by phosphatidylcholine (G, Fig. 14), because in these liposomes the lipids are in a normal monolayer association that constitute the bilayer, in consequence no immunoreaction with H308 monoclonal antibody is detected.

**Clean Copy of Page 60, Paragraph on Lines 15-20**

B64  
Aliquots of 100  $\mu$ l of patients' sera that were analyzed in Examples 1 and 2B were incubated with 100  $\mu$ l of 0.2  $\mu$ moles of glycerolphosphorylcholine for 30 min at 30°C. Later on, blocked patients sera were added to the wells of the microtiter plate and the liposomal-ELISA method was applied as it was described in Example 1. Peroxidase-conjugated goat anti-Fc of human IgG, IgA and IgM antibodies were used as second antibody.

**Clean Copy of Page 60, Paragraph on Line 30 through Page 61 Line 5**

B65  
Studies in BALB/c female mice in which were simultaneously administered the H308 monoclonal antibody, which developed in these mice a pathology similar to human antiphospholipid syndrome as it was described in Example 4C, and the glycerolphosphorylcholine hapten, showed a blockage in the development of the pathology in BALB/c female mice. H308 monoclonal antibody was administered by intraperitoneal injection of 1  $\mu$ g each week during two months to BALB/c female mice, and simultaneously the glycerolphosphorylcholine hapten was administered at 2.5 mg/Kg, of body weight, doses by

B65  
cond intravenous injection each 24 hs, for 2-months. With this treatment it was inhibited in 40% the development of mice pathology by H308 monoclonal antibody.

**Clean Copy of Page 63, Paragraph on Lines 1-3**

B66  
**Example 7A.** Study by the cytofluorometry method of the cellular membranes stabilization that prevents the formation of lipidic particles and the later binding of anti-lipidic particles antibodies.

**Clean Copy of Page 63, Paragraph on Lines 12-17**

B67  
Relative size and/or Ag4 mouse myeloma cells aggregation were analyzed in the FSC channel and the granularity or cellular membranes complexity in the SSC channel. Analysis of 10,000 Ag8 mouse myeloma cells was made with the following detectors: FSC in E00 in lineal mode with an amplifier gain of 2 V, with a detector compensation threshold of 52 V, and SSC of 250 V. The obtained data were analyzed with the Cellquest program (Beckton Dickinson).

**Clean Copy of Page 63, Paragraph on Lines 18-23**

B68  
Results obtained with Ag4 mouse myeloma cells incubated with the lipidic particles inducer drug chlorpromazine and the lipid bilayer stabilizer drugs spermidine or chloroquine were similar to those described in Figure 16 graphs. These results showed that Ag4 cellular membranes were stabilized by their interaction with lipidic bilayer stabilizers drugs spermidine or chloroquine in consequence they do not develop lipidic particles with chlorpromazine.

**Clean Copy of Page 63, Paragraph on Lines 24-28**

B69  
When Ag4 mouse myeloma cells incubated with both drugs: the lipidic particles inducer drug and the lipidic bilayer stabilizer drug were used as antigens there were not any immunoreaction with the H308 monoclonal antibody, in a similar way as was demonstrated for liposomes stabilized with the drugs spermidine or chloroquine in Example 7.

**Clean Copy of Page 63, Paragraph on Lines 29-31**

B7D These results indicate that cellular membranes of Ag4 mouse myeloma cells stabilized by their interaction with spermidine or chloroquine do not develop lipidic particles and therefore they do not react with the H308 monoclonal antibody.

**Clean Copy of Page 64, Paragraph on Lines 1-7**

B7I Examples 7 and 7A were repeated using as antigens egg-yolk phosphatidylcholine: phosphatidate (2:1 molar ratio) liposomes, in Tris-NaCl buffer (10 mM, 1 mM) pH 7, and containing 0.1  $\mu$ mol of phosphatidate, or Ag4 mouse myeloma cells. Liposomal or cellular antigens were incubated with the lipidic particles inducer drug chlorpromazine 0.2 mM for 30 min at room temperature before the treatment with the lipidic bilayers stabilizer drugs spermidine or chloroquine. Stabilizer drugs were used at the concentrations used in Example 7A.

**Clean Copy of Page 64, Paragraph on Lines 22-34**

B7E Studies in BALB/c female mice in which were simultaneously administered the H308 monoclonal antibody, which develop in these mice a pathology similar to human antiphospholipid syndrome as it was described in Example 4C, and one of the lipid bilayer stabilizer drugs spermidine or chloroquine, showed a delay in the development of this pathology. H308 monoclonal antibody was administered by intraperitoneal injection of 1  $\mu$ g each week for 2-months to BALB/c female mice, and simultaneously the stabilizer drug chloroquine was administered at 2.5 mg/Kg, of body weight, oral doses each 24 hs, during two months. With this treatment it was delayed the development of mice pathology induced by H308 monoclonal antibody. It is possible that modifying the doses of stabilizers drugs that are applied to mice it is possible to prevent the development of these illnesses. The chloroquine dose used was similar to the one used in humans in the treatment of rheumatoid arthritis and of systemic lupus erythematosus.